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AN ALTERNATIVE TO ENDOSULFAN: STRUCTURE BASED PHARMACOPHORE MODELING, GLIDE AND MM-GBSA ANALYSIS OF ECDYSONE RECEPTOR AND MOLECULAR DYNAMICS STUDIES

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ABSTRACT

Endosulfan, an insecticide which is considered as a boon to farmers to control, a host of insects that are responsible for various diseases in plants on the other hand has lethal effects on human beings as it is a carcinogen. The target of endosulfan in all the arthropods is the Ecdysone receptor which is homologous to the human Estrogen Receptor. As endosulfan is an Estrogen like endocrine disrupting chemical, it mimics estrogen and binds to the estrogen receptors causing various disorders like breast cancer and other disorders related to attainment of puberty. Phytochemical compounds tend to bind to the ER beta receptor which doesn't affect the estrogen binding. A pharmacophore was developed keeping in mind, the binding pattern of flexible endosulfan with the flexible Ecdysone receptor. The developed pharmacophore was used to screen the database of phytochemical compounds to identify the hits. These hits were docked with the Ecdysone receptor and were shortlisted based on three criteria: i) glide score ii) active site residues interactions with ecdysone receptor and iii) binding free energy. Interestingly, one of the obtained compounds named Brassinolide, was known to have insecticidal activity indicating that the developed pharmacophore model would be useful for identifying s insecticides.

KEYWORDS: Ecdysone, Endosulfan, E-Pharmacophore, MM-GBSA

INTRODUCTION

Endosulfan is a polychlorinated compound used for controlling a host of insects in Paddy, Jowar, Maize etc. Endosulfan is preferred because of its acute toxicity towards insects. It is the same acute toxicity towards mammals that resulted in the imposition of ban on the compound. Agrochemical research over the last two decades has resulted in the discovery of chemically novel insecticides that disrupt insect hormones [1]. Endosulfan, an estrogen like endocrine disrupting chemical mimics the activity of naturally occurring estrogen, which has deleterious effects in Mammals ranging from retardation in the development of gonads and to cancer in some extreme cases. [6]

Ecdysone Receptor, a nuclear receptor and transcription factor binds ecdysteroids which play an important role in moulting [2]. Estrogen receptors (ER α and ER β) induce the sex hormones with similar roles in humans. Estrogen receptors are ligand-activated transcription factors that belong to the nuclear hormone receptor super family. It consist of two hetero dimers (ER α and β) which in turn includes three domains, ligand binding domain DNA binding domain and N-terminal domain. The amino acid sequence identity between ERα and ERβ is approximately 97% in the DNA-binding domain and approximately 56% in the ligand-binding domain (LBD), whereas the N terminus is poorly homologous at 24%.[3]

Ecdysone receptor, a human homolog of the Estrogen receptor, plays an important role in the regulation of growth, development, metamorphosis and reproduction of insects. In Mammals, Estrogen receptors induce sex hormone, 17β-estradiol, which plays important roles in the growth and maintenance of a diverse range of tissues such as the mammary gland, uterus, bone and the cardiovascular system. [2,4]. Previous studies also report the structural similarity between Ecdysone receptor and ER α subunit of the Estrogen Receptor, particularly in terms of the orientations conformations of helices 1, 3, and 4.[5]

Previous studies indicate that phytochemical compounds have more affinity towards ER β than ER α. This selective binding of the phytochemical compounds to the ER β subunit of the Estrogen receptor, thus reducing the lethality of the compound binding and these compounds are called Selective Estrogen Receptor Modulators (SERMs).[7] A phytochemical compound, with similar Pharmacophoric features but a different scaffold can serve as alternative to endosulfan. Thus a pharmacophore developed for endosulfan was used to screen against the database of the phytochemical compounds to find an alternative.

METHODS

Induced Fit Docking

The 3D structure of crystallized protein with the hormone Ponasterone A in its active site was downloaded from the Protein Data Bank (PDB ID: 1R1K). The protein had two chains A and D, of which the chain A was deleted and the chain D was retained for the study as it binds Ponasterone A in its active site. Protein preparation was performed using "protein preparation wizard" of Schrodinger. The protein was then energy minimized using OPLS_2005 to obtain the least possible energy conformation using Protein preparation wizard of Schrodinger. The 3D structure of endosulfan was minimized using Ligprep. Before performing the docking studies, the receptor grid was generated by selecting the active site residues that interact with the Ponasterone A, these are Glu 309, Thr 343, Thr 346, Ala 398, Arg 383, Gln 310, Pro 311, Met 380, Val 384, Arg 387, Leu 396, Met 413, Val 416, Leu 420, Tyr 408, Ala 398, Phe 397, Met 342[8], and the docking studies were performed with endosulfan using induced fit docking. It is very important to study different conformations of the ligand and the receptor to identify the conformation with optimal interactions. Induced fit docking, which generates different conformations of protein-ligand complex serves this purpose. All these conformations were ranked based on the Glide score. The conformation with a glide score of **-6.39** was selected after analyzing the interactions between the Protein and Endosulfan.

Pharmacophore Generation

The best conformation of Ecdysone receptor-Endosulfan complex generated in the above step was used as input for E-pharmacophore option of Maestro to generate the Structure based pharmacophore model. The generated hypotheses contain information about the Pharmacophoric features and their energy values. Each Pharmacophoric feature was ranked based on the Glide XP scoring terms. The Pharmacophoric features obtained were then compared with the docking result to note the features interacting with the receptor and interacting features were exported as the best hypothesis using the option "write hypothesis with selected features".[9]

Database Screening

The above hypothesis was employed to screen a database of 2,000 KEGG phytochemical compounds. All compounds were minimized using LigPrep and conformations were generated using ConfGen algorithm. Compounds

with these conformers were then given as an input to "find matches "option to screen the database. Compounds with Pharmacophoric features similar to that of hypothesis were obtained. The compounds were ranked on the basis of fitness score. The phytochemical compounds with a fitness score above 1.5 were selected for further analysis.

Glide Docking Studies and MMGBSA

The identified hits were taken for docking studies using the Glide protocol of Schrödinger. The best docking poses were selected based on the Glide score and active site residue interactions formed by the co-crystal ligand. RMSD is one of the methods used to validate the docking program.[10] The docked poses were minimized using the local optimization feature in Prime and the energies were calculated using the OPLS 2005 force field and GBSA continuum solvent model as described previously. The MM-GBSA analysis was performed for all the shortlisted phytochemical compounds to calculate ligand binding energies and ligand strain energies for a set of ligands and a receptor **[11]**.

Molecular Dynamics Simulation Studies

Molecular dynamics simulation studies were carried out for the known and best hit compounds using Desmond of Schrodinger. The protein-ligand complex was minimized using OPLS_2005 force field. The minimized complex was then inputted for MD simulation. Before performing the MD simulation, the complex file was solvated using Single Point Charge model (SPC) in the orthorhombic box with the box size volume (14740Angstroms). The system was then neutralized with Na + ions. The system was further relaxed before the actual simulation by a series of energy minimizations and short MD simulations. Further the system was employed to 3nanoseconds molecular dynamics with NPT ensemble. The coordinates were saved at intervals of 4.8 picoseconds, referred to as 'frames' in this study. RMSD values, ROG and RMSF values were calculated using the Simulation Event Analysis module of DESMOND.

RESULTS

Induced Fit Docking

To find the stable docking pose, Induced fit docking was performed. The stable binding pose of endosulfan in the active site of ecdysone was selected from three different conformations obtained, based on the glide score and aminoacid interactions. The best scored pose had H-bond interactions with Ala 398[12]. In addition this complex is also stabilized by forming hydrophobic interactions with the aminoacid residues Phe 397, Tyr 408, the binding pose of endosulfan in the active site of ecdysone receptor is shown in **Figure 1.** As these results were highly correlated with the binding patterns of Ponasterone A in the active site of ecdysone receptor, so this binding pose of endosulfan was taken as input for designing the energy based 3D-pharmacophore. Both the molecules were observed to share similar interactions with Ala 398, Phe 397 and Tyr 408. This suggests the possibility of competitive binding of both ligands with the Ecdysone Receptor.

Pharmacophore Generation

E-Pharmacophore approach was used to generate the pharmacophore, as it is obtains interaction energies from the docked complex[9]. E-pharmacophore generated had 7 Pharmacophoric features mapping with the endosulfan. This hypothesis was validated by cross checking with docking result to see exactly whether the generated pharmacophore model mapped with bonded and non bonded interactions formed in the active site of ecdysone receptor. Out of **7** features, 4 features were well mapped with the H-bond and non bonded interactions formed by endosulfan with the ecdysone receptor. The Pharmacophoric hypothesis generated were AAHH i. e. two H-bond acceptors and two Hydrophobic features.

Mapping of Pharmacophore Features with the Active Site Residues of Ecdysone Receptor

The final hypothesis had 2 hydrophobic features (H3 & H4) and 2 H-bond acceptors (A1 & A2). The H-bond acceptor (A2) mapped well with the aminoacid Ala 398 occupying the active site of ecdysone receptor while H-bond acceptor A1 forms a negatively charged interaction with the Glu 309. The Hydrophobic feature H4 shares hydrophobic interactions with the Tyr 408 and Phe 397 whereas the H3 spanned the aminoacid residue Val 384. Hence the hypotheses generated encompasses the active site residues which will help in identifying and development of novel inhibitors that bind to the ecdysone receptor. The Pharmacophoric model developed is shown in **Figure 2.**

Pharmacophore Screening against Database of Phytochemical Compounds

The refined hypothesis was used as query to screen the database of phytochemical compounds. Molecules were shortlisted based on the fitness scores i.e. molecules with >1.5. Phase found 244 hits which mapped well with the fitness score of > 1.5. The fitness score for these hits were ranging from 1.5 to 2.0.

XP Ligand Docking

XP ligand docking was performed for the above 244 molecules with the Ecdysone Receptor, using Glide module from Maestro. The hits were shortlisted based on two criteria i) docking score (> 10.54) and ii) Ponasterone A interacting amino acids in the active site of ecdysone receptor[10]. The docking scores of phytochemical compounds that have a greater glide score **(> 10.54 Kcal/mol)** greater than that of the endosulfan are represented in the Table 1. These compounds share almost similar interactions as Ponasterone, hence these phytochemical are presumed to bind competitively with the Ecdysone receptor. The binding mode of best 4 compounds was discussed below and the images are shown in **Figure 3**.

BINDING MODES OF THE IDENTIFIED HITS

Binding Mode of Brassinolide

This compound had the highest glide score **-15.5kcal/mol** in the active site of ecdysone receptor. Brassinolide established various H-bond interactions with key aminoacid residues like Glu 309, Arg 383, Thr 346, Ala 398, and Asn 504. The oxygen atom (O2) of brassionlide moiety forms H-bond interaction with the oxygen atom of Glu 309. The oxygen atom (O2) of brassionlide also interacts with the Arg 383. The oxygen atom (O5) forms H-bond interaction with NH group of Ala 398 with the distance of 2.67Å. The oxygen atom (O3) formed close H-bond interaction with the Asn 504. Considerable hydrophobic interactions were also observed between the Brassinolide and ecdysone receptor which include Pro 311, Ile 339, Met 380, Met381, 395, Phe 397, Ala 398, 408, 413, Leu 511, Trp 526. Brassinolide is a plant hormone which protects plants from insects. This makes Brassinolide, the best candidate for the alternative to endosulfan. [13]The plant source of this compound is *Brassica napus*[14]. Brassinolide is a plant hormone, which plays a major role in plant immunity from pests. This can act as an insecticide and an alternative to endosulfan.

Binding Mode of Syringin

Syringin had gained substantial H-bond and hydrophobic interactions with the glide score of -11.90 Kcal/mol. The H-bond interactions formed by Syringin are with the residues Glu 309, Ala 398, Arg 387, Asn 504. Syringin also shares hydrophobic interactions with the Ala 398, Met 380, Met 381, Leu 420, Ile 339, and Met 342. The O3 atom of Syringin moiety forms H-bond with the aminoacid residue Ala 398. The O6 atom of Syringin shares H-bond interaction

with the side chain residues Glu 309, Arg 387 with the distance of 3.06Å and 3.19Å. Another H-bond interaction was also observed with the NH group of Asn 504. Syringin is also a natural chemical compound which was first isolated from the bark of *Syringa vulgaris*. It is also found in dandelion coffee.[15]

Binding mode of Glucosisymbrin

With a glide score of -11.01Kcal/mol the Glucosisymbrin produced H-bond and hydrophobic interactions in the active site of ecdysone receptor. Hydrogen bonding was observed between the oxygen atom (O5) of Glucosisymbrin and OG1 atom of Thr 346 aminoacid residue. H-bond interactions are also observed with the aminoacid residues Arg 383 and Glu 309 with the distance of 2.99Å and 2.49Å. Additionally, this complex is stabilized by forming hydrophobic interactions with the aminoacid residues Met 342, Pro 311, Ile 339, and Phe 397. Glucosisymbrin is found in the seeds of *Sisymbrium austriacum*[16]

Binding Mode of Provincialin

The binding mode of this hit had a Glide score of -10.93Kcal/mol in the active site of ecdysone receptor. H-bond interactions formed by this hit compound were less compared to the above hits. The oxygen atom (O10) of the ligand moiety formed bivalent H-bond interaction with the Arg 383. The O3 and O7 atom mapped with the NH group of amino acid residue Asn 504. Interestingly Provincialin also shares hydrophobic interactions with the amino acid residues Ala 398, Met 342, Val 384, and Val 395.

Binding Free Energy Analysis of Identified Hits

The binding free energy for endosulfan and above hits was observed. Interestingly Brassinolide exhibited a binding free energy of -162.101 compared to -96.865 of the endosulfan. The other compounds binding free energy were closer to that of endosulfan. This analysis indicates that the compounds retrieved from this study exhibit good binding affinity as endosulfan. Interestingly, Brassinolide was known to have insecticidal activity indicating that the developed pharmacophore model will be useful for identifying novel insecticides [11]. Thus the phytochemical compounds retrieved from this study serve as an alternative to endosulfan. The binding modes of all the compounds with their GLIDE scores, interaction energies, Binding free energies and the interactions are shown in **Table 1**.

MD Result

To identify the protein flexibility in the presence of ligand binding, two compounds namely endosulfan and best hit, Brassinolide were subjected to molecular dynamics simulation. Molecular dynamic simulation was performed in explicit aqueous solution for 3nanoseconds using DESMOND. The stability of the system was estimated by RMSD. All the analyses were performed using the Simulation Event Analysis (SEA) tool. The RMSD plot suggests that the backbone RMSD for ligand endosulfan had significant stability approximately for 1.5 nanoseconds and suddenly diverged and reaches equilibrium with the average fluctuation of 1.1. The |RMSD for Brassinolide was nearly significant over the course of 2.6 nanoseconds and reaches equilibrium with the average fluctuation of 0.9 RMSD. The analysis of the average RMSD of the compounds endosulfan and Brassinolide in 3 nanoseconds MD simulation was calculated from the initial structures. RMSD of endosulfan was 0.248Å and for Brassinolide 0.180Å indicating that the protein was found to be fairly stable with the Brassinolide than the endosulfan. The Molecular Dynamics result is shown in **Figures 4 and 5.**

RMSF

RMSF was plotted to find the fluctuations in the areas of active site aminoacid residues in the presence of ligand molecules over the 3 nanosecond simulation. The analysis of root-mean-square fluctuation (RMSF) versus the residue number for both ligands is illustrated in Figures 4 and 5. It can be seen that there are four major flexible segments in the protein. These lay in the corresponding residues 315-323,355-365,454-480 in the endosulfan (blue) complex. These fluctuations are higher in the endosulfan bound protein complex than the Brassinolide bound complex. The active site residues **Glu 309, Thr 346, Val 384, Phe 397, Ala 398 and Asn 504** have larger conformational drift with the endosulfan bound complex than the Brassinolide. The average conformation of the protein active site residues from MD simulation was superimposed on the docked conformation of the two complexes. The average conformation of the active site residues in the 3 nanoseconds MD simulation with the endosulfan was a 1.04 Å, whereas for Brassinolide 0.735Å. The active site residues were relatively crimped for the endosulfan bound complex this is because of the change in conformation of active site residues like Phe 397, Thr 346and Glu 309. Whereas the in the presence of other ligand, Brassinolide the change in conformation of active site residues were very less. To evaluate the stability of these interactions distances between ligands and protein active site residues were observed using simulation event analysis of Desmond.

Brassinosteroids are a class of plant secondary metabolites that play a major role in the innate immunity of the plants. This property of Brassinosteroid hormones can be exploited for their insecticidal activity. Moreover, Btrassinolide has the pharmacophoric features of Endosulfan, which makes it more preferred alternative for Endosulfan. Campesteol, oxatyphasterol, castasterone are the immediate precursors in Brassinolide synthesis pathway. CYP85A2 gene plays an important role in the synthesis of Brassinosteroids.[17]

CONCLUSIONS

It has been established that Endosulfan, binding to the human Estrogen Receptor has lethal effects. Phytochemical compounds act as Selective Estrogen Receptor Modulators which selectively bind to the different sub units of the Estrogen Receptor. This leaves us with a scope of developing a phytochemical alternative to Endosulfan. Structure based Pharmacophoric approach was employed in this study due to its advantages over the ligand based approach. An efficient pharmacophore with 4 features 2 hydrophobic features (H3 & H4) and 2 H-bond acceptors (A1 & A2) was developed and was employed for screening against the database of phytochemical compounds. The screening produced more than 250 compounds with a fitness score of 2 or above. These compounds were shortlisted using docking. The compounds' docking scores and the binding patterns of the receptor were analyzed. The short listed compounds had similar binding patterns to that of Ponasterone hormone and hence they may serve as alternatives of lethal endosulfan. The shortlisted compounds interact with the receptor with the residues that interact with Ponasterone A i.e. Asn 504, Val 379, Glu 309, Met 502, Arg 498, Thr 343, Thr 346, Gln 310, Thr 320. Post docking validation was also performed using MMGBSA for four compounds to find out the binding affinities. Further MD studies proved that Brassinolide had enhanced stability with ecdysone recept or compared to the known ligand endosulfan.

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17. KEGG Pathway database ,

APPENDICES

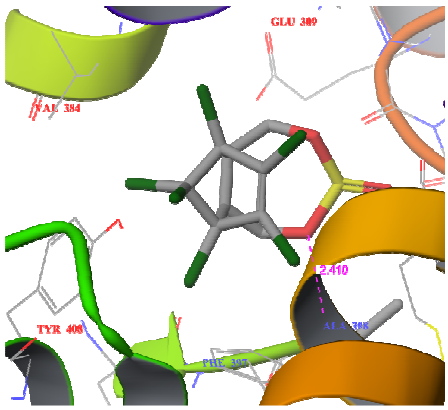

Figure 1: Binding Pose of Endosulfan in the Active Site of Ecdysone Receptor

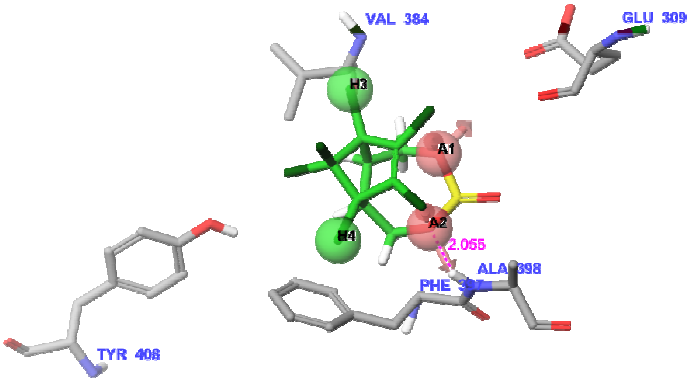

Figure 2: The Pharmacophore Developed with Two Hydrogen Bond Acceptors and Hydrophobic Features

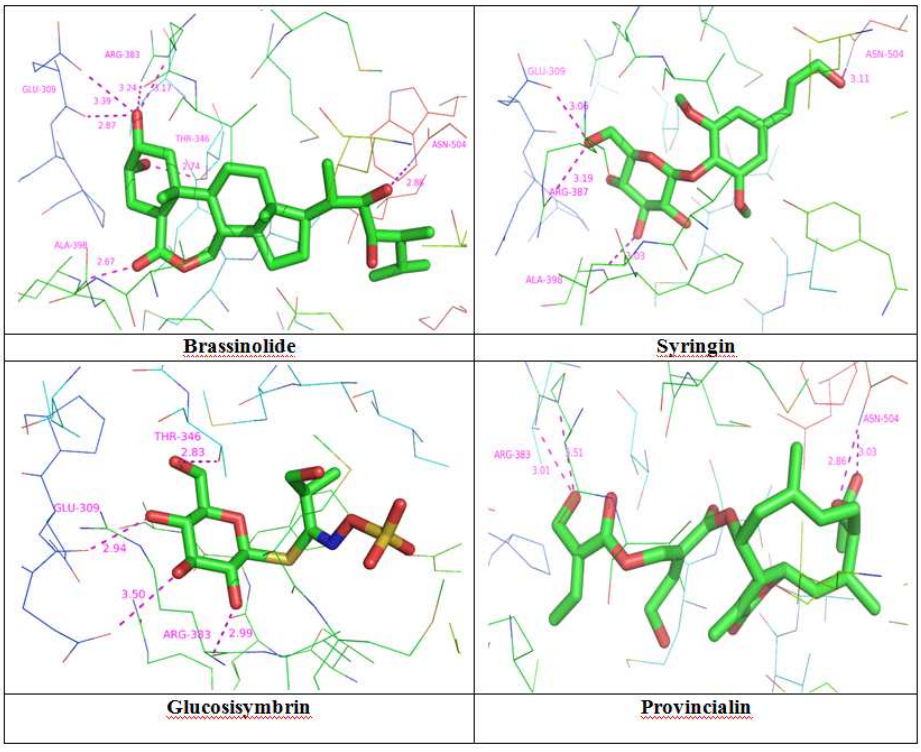

Figure 3: Binding Modes of Brassinolide, Syringin, Glucosisymbrin and Provincialin Molecules

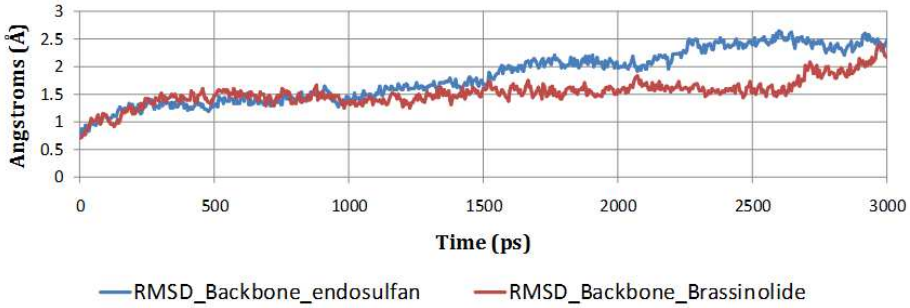

Figure 4: RMSD Studies of Endosulfan and Brassinolide in the Active site of Ecdysone Receptor

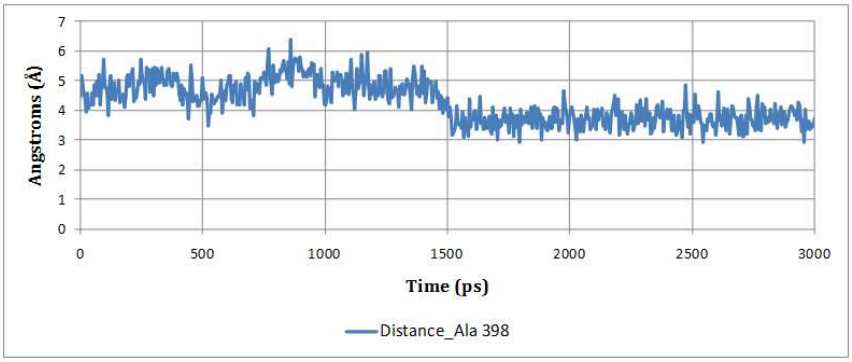

Figure 5: Root Mean Square Fluctuation Graph

Table1: Docking Scores, Energies, Binding Free Energy, H-bond and Hydrophobic Interactions of Phytochemical Compounds

Compound Name	Glide Score (Kcal/mol)	Glide Energy (Kcal/mol)	MM-GBSA	H-Bonds	Hydrophobic
Endosulfan	-6.39	-21.163	-96.865	Ala 398	Tyr 408, Phe 397
Brassinolide	-15.53	-61.402	-162.101	Glu 309, Arg 383, thr 346, ala 398, asn 504	Pro 311, Ile 339, Met 380, Met381, 395, Phe 397, Ala 398, 408, 413, Leu 511, Trp 526
Syringin	-11.90	-48.491	-64.905	Glu 309, ala 398, arg 387, asn 504	Ala 398, Met 380, Met 381, leu 420, Ile 339, Met 342
Glucosisymbrin	-11.01	-47.935	-45.107	Glu 309, Thr 346, arg 383,	Met 342, Pro 311, Ile 339, Phe 397
Provincialin	-10.93	-43.889	-35.107	Arg 383, asn 504	Ala 398, Met 342, Val 384, Val 395